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SINGLE CELL STUDIES OF HUMAN T CELL FUNCTION WITH APPLICATIONS FOR HIV-1

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SINGLE CELL STUDIES OF HUMAN T CELL FUNCTION WITH APPLICATIONS FOR HIV-1

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family

ABSTRACT

T cells are one of the body's main defenses against viruses and cancers and are therefore considered to play a major role in immunotherapies after stem cell transplantation and in HIV-1 vaccine and cure strategies. However, malignancies and chronic viral infections, such as HIV-1, eventually cause the T cells to become dysfunctional, resulting in a loss of control. As the T cell population is highly heterogeneous, studying the characteristics of the relatively few cells that are specifically recognizing infections and malignancies is of immense importance to fully understand what makes up an effective T cell response. Therefore, the aim of this thesis has been to evaluate the single-cell characteristics of T cells using microwell-chip based imaging and multi-color flow cytometry.

The cytokine IL-2 is widely used to expand T cells for immunotherapy, but it also leads to expansion of the regulatory T cells (Tregs) that dampen the desired T cell response. We evaluated the effects of addition of IL-7, which previously was shown to decrease Treg expansion, to the protocol by monitoring the actions of T cells with single-cell resolution in a microwell chip. Addition of IL-7 did not affect the migration properties or cell-cell interactions, however it increased T cell survival in the microwell chip. Overall, the microwell chip was proved to be suitable for T cell studies and addition of IL-7 was confirmed to be beneficial when preparing T cells for immunotherapy.

During HIV-1 infection, $CD8^+$ T cells become exhausted, *i.e.* upregulate inhibitory receptors in a process linked to the loss of functional properties, due to the constant antigen burden. We found that the transcription factors T-bet and Eomes were inversely expressed on bulk and HIV-specific $CD8^+$ T cells. Cells with a high expression of Eomes and a low expression of T-bet were linked to an increased expression of inhibitory receptors, a transitional memory phenotype and a loss of functional capacity. This transcriptional profile remained after more than 10 years of treatment, suggesting that this might contribute to an inability of HIV-specific $CD8^+$ T cells to control the infection even during successful treatment.

We continued by investigating the role of a novel inhibitory receptor, T cell immunoglobulin and ITIM domain (TIGIT), on $CD8^+$ T cell exhaustion during HIV-1 infection. TIGIT was upregulated on bulk and HIV-specific $CD8^+$ T cells and was linked to an increased expression of markers of exhaustion and immune activation as well as a T-$bet^{dim}$$Eomes^{hi}$ transcriptional phenotype. Furthermore, upregulation of TIGIT on HIV-specific $CD8^+$ T cells was linked to the downregulation of its complementary co-stimulatory receptor CD226 and a diminished functional capacity. Finally, expression of the TIGIT/CD226 ligand PVR was upregulated of T follicular helper cells, representing a major source of latent and productive HIV-1 infection. The result suggests that PVR is upregulated on HIV-infected cells and provides another major obstacle for HIV cure strategies.

LIST OF SCIENTIFIC PAPERS

I. **Tauriainen J,** Gustafsson K, Göthlin M, Gertow J, Buggert M, Frisk TW, Karlsson AC, Uhlin M, Önfelt B. Single-Cell Characterization of in vitro Migration and Interaction Dynamics of T cells expanded with IL-2 and IL-7. *Front Immunol. 2015 Apr 28;6:196. doi: 10.3389/fimmu.2015.00196*

II. Buggert M, **Tauriainen J,** Yamamoto T, Fredriksen J, Ivarsson MA, Michaëlsson J, Lund O, Hejdeman B, Jansson M, Sönnerborg A, Koup RA, Betts MR, Karlsson AC. T-bet and Eomes are differentially linked to the exhausted phenotype of CD8+ T cells in HIV infection. *PLoS Pathog. 2014 Jul 17;10(7);e1004251. doi:10.1371/journal.ppat.1004251*

III. **Tauriainen J**, Fredriksen J, Scharf L, Ljunggren HG, Sönnerborg A, Naji A, Del Rio P, Reyes Terán G, Lund O, Hecht FM, Deeks SG, Betts MR, Buggert M, Karlsson AC. Inverse Expression of TIGIT and CD226 on HIV-specific $CD8^+$ T cells in Linked to Impaired T cell Function and Increased Expression of PVR on $CD4^+$ T cells. *Manuscript. 2016*

LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

i. Norström MM, Buggert M, **Tauriainen J**, Hartogensis W, Prosperi MC, Wallet MA, Hecht FM, Salemi M, Karlsson AC. Combination of immune and viral factors distinguishes low-risk versus high-risk HIV-1 disease progression in HLA-B*5701 subjects. *J Virol. 2012 Sep;86(18):9802-16. doi: 10.1128/JVI.01165-12*

ii. Buggert M, Frederiksen J, Lund O, Betts MR, Biague A, Nielsen M, **Tauriainen J**, Norrgren H, Medstrand P, the SWEGUB CORE group, Karlsson AC, Jansson M. $CD4^+$ T cells with an activated and exhausted phenotype distinguish progressive disease during aviremic HIV-2 infection. *Submitted to AIDS. 2016*

CONTENTS

LIST OF ABBREVIATIONS

ADCC	Antibody dependent cellular cytotoxicity
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
ART	Antiretroviral treatment
bNAb	Broadly neutralizing antibody
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte associated protein-4
CRF	Circulating recombinant form
DLI	Donor lymphocyte infusion
DNA	Deoxyribonucleic acid
DNAM-1	DNAX accessory molecule-1
EC	Elite controller
Env	Envelope
Eomes	Eomesodermin
Gag	Group specific antigen
HDACi	Histone deacetylase inhibitor
HSCT	Hematopoietic stem cell transplantation
HIV-1	Human immunodeficiency virus type 1
HLA	Human leukocyte antigen
IFN-γ	Interferon gamma
IL	Interleukin
IN	Integrase
ITIM	Immunoreceptor tyrosine-based inhibition motif
MHC	Major histocompatibility complex
Nef	Negative regulatory factor
NK	Natural killer

NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
Pol	Polymerase
PR	Protease
PBMC	Peripheral blood mononuclear cell
PCA	Principal component analysis
PD-1	Programmed death-1
PD-L1	Programmed death ligand-1
PDMS	Polydimethylsiloxane
PI	Protease inhibitor
PVR	Poliovirus receptor
Rev	Regulator of virion
RNA	Ribonucleic acid
RT	Reverse transcriptase
SPICE	Simplified presentation of incredibly complex evaluations
SIV	Simian immunodeficiency virus
TCR	T cell receptor
Tat	Transcriptional transactivator
Th	T helper
Tfh	T follicular helper
TIGIT	T cell immunoglobulin and ITIM domain
TNF	Tumor necrosis factor
Treg	T regulatory cell
Vif	Virion infectivity
Vpr	Viral protein R
Vpu	Viral protein U
VL	Viral load

1 INTRODUCTION

1.1 INTRODUCTION TO T CELLS

T cells, or T lymphocytes are cells of the adaptive immune system. All T cells express the T cell receptor (TCR), which recognizes antigens, i.e. peptides cleaved from proteins that can be either self-derived or derived from pathogens or malignant processes. These peptides are presented by the major histocompatibility complex (MHC) molecules (in humans called human leukocyte antigen (HLA)). The two main groups of T cells are the $CD8^+$ T killer cells (also called cytotoxic T cells (CTL)) and the $CD4^+$ T helper cells. The TCR alone is not enough for T cells to recognize antigen presented by HLA molecules, in addition a co-receptor is needed to stabilize the TCR. For $CD8^+$ T cells this is the cluster of differentiation 8 (CD8) molecule, which together with the TCR recognizes antigens presented by HLA class I molecules, which are present on all nucleated cells in the body (Fig. 1). In turn, $CD4^+$ T cells express the CD4 molecule that together with the TCR recognizes antigen presented on HLA class II molecules that are present on professional antigen presenting cells (APC), such as dendritic cells, monocytes and B cells. The HLA-molecules are the most polymorphic genes in the human body. The HLA class I gene is further divided into HLA-A, -B and -C alleles and the HLA class II gene can be further divided into HLA-DP, -DQ and -DR. Each allele is in turn present in different isoforms, making sure that the T cells are able to combat a broad variety of pathogens.

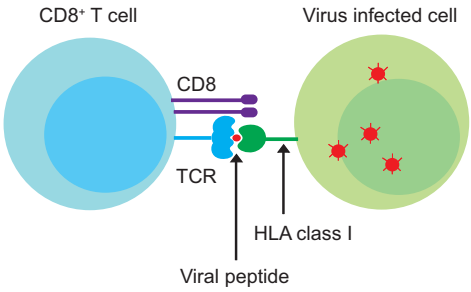

Figure 1. $CD8^+$ T cell recognition of an infected cell. The TCR, together with the CD8 molecule on the $CD8^+$ T cell recognizes a viral peptide presented by a HLA class I molecule on the surface of the infected cell. TCR: T cell receptor; HLA: Human leukocyte antigen; CD8: Cluster of differentiation 8.

1.1.1 T cell development

All T cells arise from the same common ancestor stem cells in the bone marrow and then migrate to the thymus where they undergo maturation. The maturation process involves T cell education where the cells are required to pass two tests. First is the positive selection, which ensures that the TCR is able to recognize HLA molecules. Cells that fail this test will not receive the necessary survival signals and will die by neglect. The second test is the negative selection, during which the TCR is presented with a HLA molecule presenting a self-derived antigen. If the cell reacts to this complex too strongly there is a risk it will cause autoimmune reactions, therefore these cells are deleted. After passing both of these tests, the T cell

"graduates" and is allowed to leave the thymus and enter the lymphatic tissues as a naïve T cell in order to scan the body for foreign antigens.

1.1.2 CD8$^+$ T cells

CD8$^+$ T cells are the killer cells of the adaptive immune system. They recognize peptides presented by HLA class I molecules on cells infected with intracellular pathogens such as viruses and cancer cells. Naïve CD8$^+$ T cells that have not encountered their cognate antigen circulate through the secondary lymphoid tissues; lymph nodes, mucosa-associated lymphoid tissue (MALT), gut-associated lymphoid tissue (GALT), tonsils, spleen and Peyer's patches. In these tissues the naïve CD8$^+$ T cells make contacts with professional APCs and when encountering their specific antigen, they receive signals to proliferate and mature into effector T cells that can be either short-lived effector cells or long-lived memory precursor effector cells (reviewed in [1]). These cells leave the lymphoid tissue and travel through the blood into the site of the infection. Here, the CD8$^+$ T cells form immune synapses with the infected target cells, through which they secrete granules containing perforin and granzymes. Perforin creates a pore in the surface of the target cell allowing for granzyme B to enter the cell and interact with caspases that cause the target cell to undergo apoptosis, thus eliminating the infected cell (reviewed in [2]). Furthermore, CD8$^+$ T cells produce a number of cytokines that are essential for containment of an infection such as interferon gamma (IFN-γ), tumor necrosis factor (TNF) and interleukin-2 (IL-2). When an infection is cleared most virus-specific CD8$^+$ effector cells die by apoptosis and less than 10% of the cells remain to create a pool of long-lived memory cells ready to quickly become activated in case of reinfection [3] (Fig. 2).

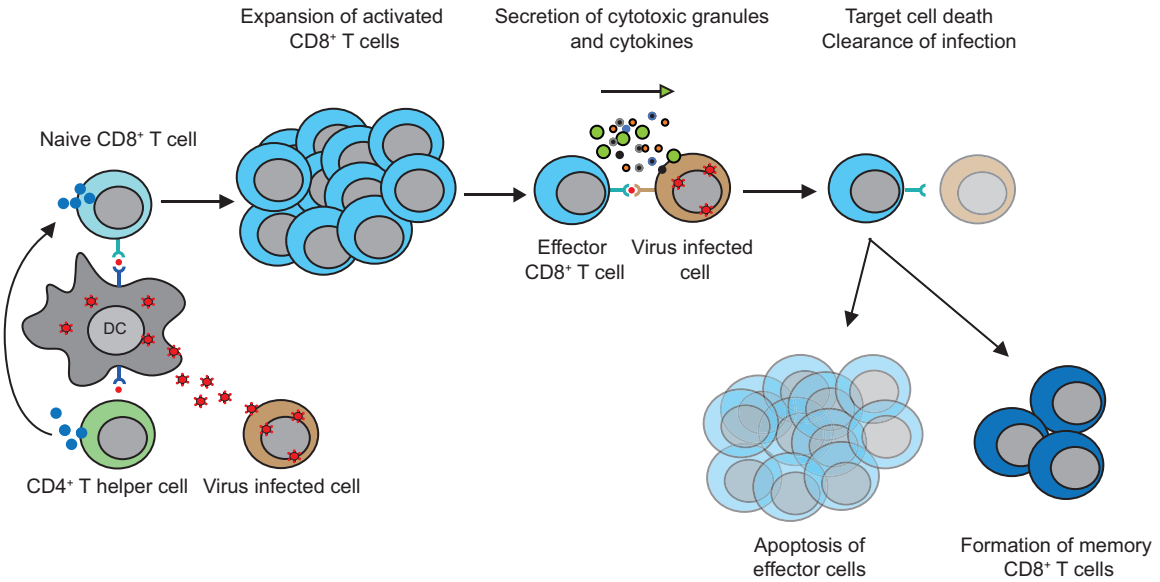

Figure 2. CD8$^+$ T cell response against a virus. When a naïve CD8$^+$ T cell recognizes its cognate peptide presented by a dendritic cell (DC), it becomes activated and starts maturing and dividing to produce a massive number of effector CD8$^+$ T cells. The effector CD8$^+$ T cells recognize infected cells and secrete cytotoxic granules in order to kill the infected cells and clear the infection. When the antigen is eliminated the effector cells are no longer needed and the majority die by apoptosis whereas a small fraction form a memory CD8$^+$ T cell pool.

Several cell surface markers are used to define the memory phenotype of T cells; CD45RO and/or CD45RA which are isotypes of the CD45 molecule that augments signaling through the TCR, the lymph node homing marker CCR7 and the co-stimulatory receptors CD27 and CD28 [4, 5]. In this thesis we have used CD45RO, CCR7 and CD27 to determine the phenotype of T cells (Table 1).

	Naive	CM	TM	EM	Eff	CD27+
CD45RO	-	+	+	+	-	-
CCR7	+	+	-	-	-	-
CD27	+	+	-	-	-	+

Table I. $CD8^+$ T cell maturation phenotypes based on expression of CD45RO, CCR7 and CD27. CM: central memory; TM: transitional memory; EM: effector memory; Eff: terminally differentiated effector; CD27+: CD27 single positive.

1.1.3 $CD4^+$ T cells

$CD4^+$ T cells, also called T helper cells are as their name implies involved in helping other cells of the immune system to combat pathogens. The $CD4^+$ T cells come in many different flavors including; Th1, Th2, Th17, T regulatory cells (Tregs) and T follicular helper (Tfh) cells, and their functions will be described below.

The $CD4^+$ Th1 cells drive the adaptive immunity pathway that combats intracellular antigens and cancer mainly by production of IFN-γ, IL-2 and IL-12 whereas $CD4^+$ Th2 cells drive the humoral immunity pathway that promotes antibody production by B cells and responses against extracellular pathogens, such as parasites and bacteria, through production of IL-4, IL-5 and IL-10 [6]. Tfh cells are another type of T helper cells that are defined by their expression of the transcription factor Bcl-6, the chemokine receptor CXCR5, and a high expression of PD-1 [7, 8]. CXCR5 allows the Tfh cells to enter the B cell follicles of the lymph node, where T cells generally do not have access, in order to aid B cell development and immunoglobulin (Ig) production [7, 9]. $CD4^+$ Th17 cells are pro-inflammatory cells involved in maintenance of mucosal barriers and in pathogen clearance in the mucosa [10]. The Tregs suppress the immune response by producing anti-inflammatory cytokines such as IL-10, TGF-β and IL-35 and by cytolysis of target cells [11]. These cells are of importance when it comes to switch off the immune response after a cleared infection to prevent the immune system from damaging healthy cells.

1.1.4 Regulation of T cells

T cell responses need to be regulated by different signals in order to match the situation the immune system is faced with. This is achieved by activating/inhibitory receptors and transcription factors.

1.1.4.1 Activating and inhibitory signals

Binding of the TCR to a HLA:peptide complex results in a signaling cascade that in the end stages induces transcription factors that regulate gene expression. In addition to TCR/HLA:peptide binding, a second activating signal is required for sufficient signaling. This is provided by co-stimulatory receptors such as CD28, CD27 [12], ICOS, CD40L [12] and CD226 (DNAM-1) [13] that signal to the T cell to proliferate, produce cytokines and promote survival in order to combat the pathogen.

After the clearance of an infection, the immune responses need to be turned off in order to prevent damage to the surrounding tissues. This is regulated by co-inhibitory receptors, which can inhibit TCR signaling. The most studied ones are programmed death-1 (PD-1) and cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) (reviewed in [14]). Other co-inhibitory receptors involved in regulation of T cells are LAG-3, Tim-3, BTLA, CD160, 2B4 (CD224) ([14]) and T cell immunoglobulin and ITIM domain (TIGIT) [15, 16]. The exact mechanisms by which these co-inhibitory receptors dampen immune responses are not known but studies have shown that they can induce inhibitory genes and modulate T cell signaling downstream of the TCR. Some inhibitory receptors such as CTLA-4 and TIGIT share a ligand with their co-stimulatory counterparts, CD28 and CD226 respectively, and can outcompete the co-stimulatory receptor for binding to the ligand [16, 17].

Both co-stimulatory and co-inhibitory receptors are required in order to retain a balance in the immune system and avoid damage to the tissues or autoimmune reactions. This delicate balance is of key to maintain a functional immune system, something that is put out of play during chronic viral infections and cancer where the antigen persists.

1.1.4.2 Interleukins

Interleukins are a group of cytokines of the gamma-chain cytokine family. They have a number of different functions on the cells of the immune system, which they exert when binding to their cognate receptors on cells. IL-2, IL-7 and IL-15 are considered the central regulators of T cell populations as they all induce proliferation and promote survival of different T cell subsets [18].

IL-2 is a T cell growth factor, produced by T cells in order to induce their proliferation. It has an important role on Treg proliferation and thus in the maintenance of peripheral tolerance. IL-2 binds to the IL-2 receptor (IL-2R), which is upregulated by IL-2, IL-7 and IL-15 as well as by antigen exposure [18].

IL-7 is involved in expansion and maintenance of T cells both in the periphery and in the thymus. It is constitutively produced by stromal cells in the lymphoid tissues. The IL-7R (CD127) is expressed on mature naïve T cells in peripheral blood and thus helps maintain the homeostasis of naïve T cells. Central memory T cells expressing the IL-7R are maintained only through IL-7R signaling without the requirement for TCR signaling thus maintaining the memory T cell pool [18].

IL-15 expands the effector memory T cells. Unlike IL-2 and IL-7 that are always secreted, IL-15 can also function through a membrane-bound isoform. Furthermore the IL-15R can be shed from cells and to block the active cytokine, making IL-15 use in immunotherapies rather complicated [18].

There are a number of other cytokines of importance for T cell functions. For example, IL-21 induces proliferation of T cells, whereas IL-4 induces differentiation of $CD4^+$ T cells into Th2. IL-17 is produced by Th17 cells and induces cytokine production and has a pro-inflammatory function. IL-23 in turn induces proliferation of Th17 cells. Furthermore, there are cytokines with immune suppressing functions, such as IL-10 that inhibits cytokine synthesis and suppresses macrophage functions and IL-35 that has an immunosuppressive function [18].

1.1.4.3 Transcription factors

Transcription factors are nuclear proteins involved in the process of gene transcription. For $CD8^+$ T cells, the T-box transcription factors T-bet and Eomesodermin (Eomes) are of key importance, although additional transcription factors are also implicated for T cell development such as BLIMP1, NFAT, BATF, VHL, FOXO1 and FOXP1 [19]. The function of T-bet is to regulate the formation of terminally differentiated effector cells during an acute infection, whereas Eomes is responsible for generating the central memory cell pool in the end stages of the infection [19]. However, when an infection becomes chronic T-bet expression decreases whereas Eomes expression increases, resulting in a population with low T-bet and high Eomes expression ($T\text{-}bet^{dim}Eomes^{hi}$) that is associated with increased expression of inhibitory receptors and decreased functionality. In contrast, the more functional $T\text{-}bet^{hi}Eomes^{dim}$ population, associated with a lower expression of inhibitory receptors ([19] & **Paper II)** decreases during chronic infection. Interestingly, T-bet has been shown to repress PD-1 expression by binding to the PD-1 regulatory region, and to regulate expression of LAG-3 and CD160 [20]. Thus the imbalance in the expression of these transcription factors during chronic viral infections could partially explain why the $CD8^+$ T cells fail to contain chronic viral infections.

1.2 T CELLS IN DISEASE

The fields of HIV and cancer research might be viewed as two widely separate fields of research, but there are significant similarities between the two. Both in HIV infection and cancer, T cells have a very important role in eradicating the virus or the malignant cells, but fail to do so and eventually become dysfunctional. This part will first give a brief introduction to hematopoietic malignancies and in the second part a more detailed description of HIV infection.

1.2.1 Hematopoietic malignancies

It is beyond the scope of this thesis to go into the field of hematopoietic malignancies in detail, but as **Paper I** deals with expansion protocols of T cells for immunotherapies after hematopoietic stem cell transplantation, a brief introduction will be given to this field.

1.2.1.1 Hematopoietic malignancies

Hematopoietic malignancies are cancers that affect the cells of the immune system. This group involves leukemia, Hodgkin's lymphoma, Non-Hodgkin's lymphoma, multiple myeloma and a number of less common blood cancers. They are a result of an abnormal differentiation of white blood cells resulting in a high number of malignant cells, which enter the blood. In order to replace the malignant cells hematopoietic stem cell transplantation (HSCT) is required.

1.2.1.2 Hematopoietic stem cell transplantation

During a HSCT the malignant blood cells of the patient are replaced with cells from a healthy donor that genetically matches the recipient [21, 22]. The first step of a HSCT is to treat the patient with radiation- and/or chemotherapy in order to clear the majority of the tumor cells and to create space for donor cells. After the initial treatment the patient receives stem cells from a donor. If the transfusion is successful, the donor's immune system will be accepted by the host's immune system and finally replace it entirely. However, HSCT is always associated with risks; the host cells can attack the foreign donor cells (host-versus-graft reaction) or the donor's immune cells attack the host (graft-versus-host reaction) (reviewed in [23]). Both reactions are potentially life threatening. Therefore, patients receive immunosuppressive therapy before and after the transplant in order to avoid these reactions. Unfortunately this, in combination with the fact that it takes time for the new immune cells to establish in the host, leaves the patient vulnerable for opportunistic infections which might be life threatening. Therefore, strategies are needed to restore the immune system of the transplantation recipients. Among these are transfusions of T cells that can combat the pathogens causing opportunistic infections. These cells can be derived from the patient's own cells (autologous) or from a genetically matched donor (allogeneic), which will be discussed in detail in section 1.3.

1.2.2 Human immunodeficiency virus

1.2.2.1 The history of HIV

In the early 1980's clinicians in the United States noticed an increase in the occurrence of lethal cases of opportunistic diseases and rare forms of cancer in a group of previously healthy patients [24, 25]. The disease was named acquired immunodeficiency syndrome (AIDS) [26]. The causative agent of AIDS remained unknown until 1983 when human immunodeficiency virus type 1 (HIV-1) was isolated from an AIDS patient [27, 28]. Later on, the origins of HIV-1 were traced back to a strain of simian immunodeficiency virus (SIV) found in West Central African chimpanzees [29]. Transmission of SIV from monkeys to

humans likely occurred in the beginning of the 20th century [30] resulting in the ongoing HIV-1 epidemic, which was fueled by the increased mobility and traveling habits of humans in the later part of the 20th century. Human immunodeficiency virus type 2 (HIV-2), a less virulent and less transmissible strain of HIV, was identified in 1986 [31]. HIV-2 was transmitted to humans from sooty mangabeys during the first half of the 20th century [32, 33]. HIV-2 is mainly present in parts of West Africa and in Portugal that have colonial ties to West-Africa, however HIV-1 remains the major cause of the epidemic.

HIV-1 is divided into the subgroups M (major), O (outlier), N (non-M/non-O) and P [34]. Of these the M group is further divided into the subtypes A, B, C, D, F, G, H, J, K and a number of circulating recombinant forms (CRFs) [35]. Subtype C is the major cause of the epidemic in many parts of Africa, whereas subtype B is the dominant form in Western Europe, the Americas and Australasia and CRF01_AE and subtype B are the dominant subtypes in Asia [36].

* The term "HIV" will be used to refer to HIV-1 throughout this thesis unless otherwise stated

1.2.2.2 Epidemiology of HIV

According to the 2015 UNAIDS report [37] 36.9 million people are currently estimated to be living with HIV and of these approximately 15.8 million receive treatment. Due to recent successes in antiretroviral treatment (ART) strategies and major prevention programs in affected countries, the number of new HIV infections is steadily decreasing in many countries. However, in the Middle East, North Africa, Eastern Europe and Central Asia there has been an alarming development of increased numbers of new HIV infections since 2000. Worldwide, 2 million people were newly infected with HIV and 1.2 million died of AIDS-related causes in 2014. Furthermore, according to the report it is estimated that 17.1 million people living with HIV do not know their HIV-positive status, which is a major risk factor for spread of the disease [37].

1.2.2.3 The HIV genome

HIV is a lentivirus belonging to the family of retroviruses. The main characteristic of retroviruses is the insertion of their genetic material into the host cell DNA, after which the cell will produce new virus particles as a part its normal activity. The genetic material of HIV consists of two single strands of positive-sense RNA that encode nine genes which can be divided into three groups; the retrovirus specific genes; *gag, pol, env,* the essential regulatory proteins *tat* and *rev* and the accessory regulatory proteins *nef, vpr, vif,* and *vpu* [38].

The group-specific antigen (*gag*) gene encodes for the Gag p55 protein, which is further cleaved into structural proteins; the capsid protein Gag p24, the matrix protein Gag p17, the nucleocapsid Gag p7 and the spacer peptide Gag p6. The polymerase (*pol*) gene encodes for the enzymes reverse transcriptase (RT) that transcribes viral RNA to DNA, integrase (IN) that integrates the viral DNA into the host genome and protease (PR) that cleaves viral

polyproteins into mature functional proteins. The envelope (*env*) gene encodes for the glycoprotein gp160, which is cleaved in order to produce the envelope proteins gp120 and gp41 that make up the viral spikes present on the virion surface that are involved in the insertion of the virus into a host cell. The HIV genome also consists of two essential regulatory proteins: transcriptional transactivator (*tat*) and regulator of virion gene expression (*rev*), which are important for viral gene expression. Additionally, there are four accessory regulatory proteins: negative regulatory factor (*nef*), viral protein r (*vpr*), viral protein u (*vpu*) and viral infectivity factor (*vif*) that are primarily involved in pathogenicity and immune evasion [38].

1.2.2.4 Structure of HIV

The HIV RNA is surrounded by the viral capsid, which also contains the viral enzymes RT, IN and PR. The matrix that lines the surface of the virion further stabilizes the virus structure. The surface of the virion is composed of a lipid bilayer, taken from the host cell, together with the viral proteins gp120 and gp41 that form spikes on the envelope and are involved in HIV entry into target cells [38] (Fig. 3).

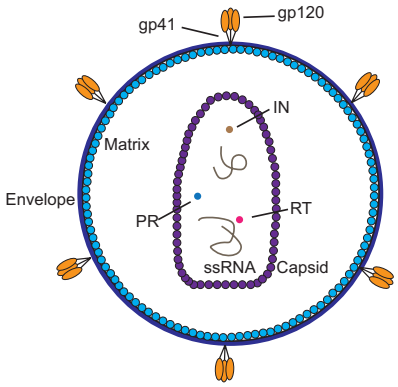

Figure 3. The structure of HIV-1. The HIV-1 RNA and the viral enzymes are surrounded by the viral capsid that in turn is contained within a lipid bilayer lined by the matrix. The surface of HIV-1 is covered with viral spikes made up by the gp120 and gp41 proteins. RT: reverse transcriptase; PR: protease; IN: integrase, ssRNA: single-strand RNA.

1.2.2.5 HIV life cycle

HIV enters host cells expressing the CD4 receptor, which is present on $CD4^+$ T cells, dendritic cells, macrophages and brain microglia. In addition, HIV requires expression of a co-receptor (CCR5 or CXCR4) in order to enter a cell [39, 40]. The process begins when the HIV protein gp120 binds to the CD4 receptor and undergoes a conformational change that allows insertion of gp41 to into the host cell membrane (Fig. 4). This enables the virion to fuse with the host cell and to release the viral nucleocapsid containing viral RNA, enzymes and molecules into the cytoplasm. HIV RNA is transcribed by the viral RT into DNA that is transported to the nucleus where it is integrated into host DNA by use of the viral protein IN. After this, HIV RNA and proteins are produced as a part of the host cell's normal machinery. In order to produce new viral particles, the viral RNA and proteins are assembled near the

cell surface after which the virus buds out of the cell, taking a part of the cell's membrane with it. The maturation process continues after the virion has left the cell as the gag-pol polyprotein is cleaved by protease in order to create a fully mature virion that is ready to infect a new host cell [38] (Fig. 4).

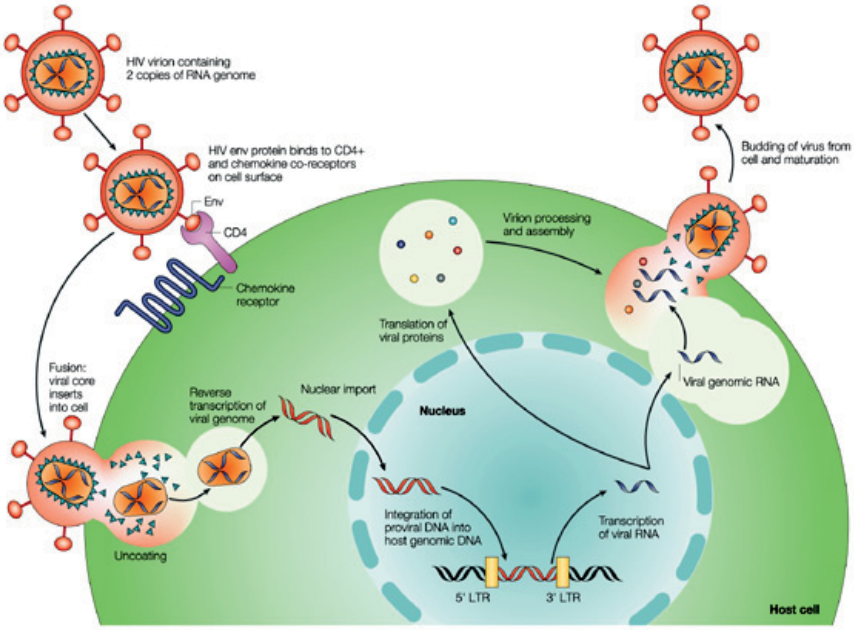

Figure 4. HIV life cycle. Reprinted with permission from [41].

1.2.2.6 HIV disease progression

HIV is mainly transmitted via blood through a sexual route, intravenous drug use, mother to child (during pregnancy, delivery or breastfeeding) and transfusion of blood components. The virus is also present in semen and vaginal fluid, although at lower levels than in blood [42, 43].

HIV disease progression is divided into three stages; acute infection, chronic infection and AIDS (Fig. 5). During the acute phase, there is an intense production of virus resulting in a peak in the viral load (VL) in blood. It is during this phase that some patients get flu-like symptoms [44]. The $CD8^+$ T cell response against HIV appears approximately three weeks after the initial infection coinciding with a decrease in the VL [45, 46] that finally reaches a plateau, called the viral set point [47]. During this time the first antibodies targeting HIV are produced, however in contrast to $CD8^+$ T cell responses, they have very little impact on viremia during this phase [48]. This is the start of the chronic phase, a long-lasting asymptomatic phase of HIV infection during which the virus slowly replicates within $CD4^+$ T cells, causing a gradual loss of $CD4^+$ T cell numbers. When the number of $CD4^+$ T cells reach below 200 $CD4^+$ T cells/μL the immune system collapses causing a severe immunosuppression, leaving the patient at risk for opportunistic infections which mark the AIDS phase [44]. Fortunately, the number of HIV-positive subjects receiving ART increases

every year, resulting in that the frequency of HIV-positive subjects reaching the AIDS phase is steadily decreasing [37].

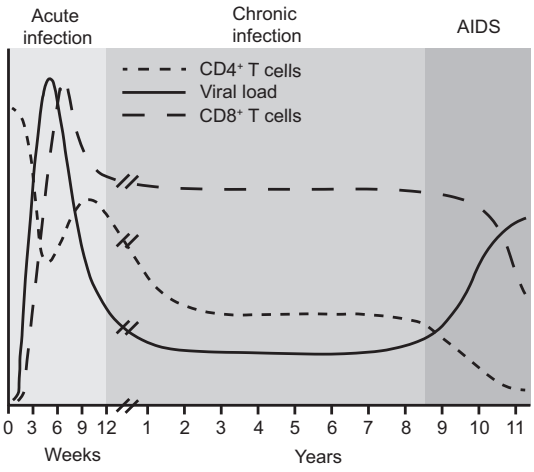

Figure 5. HIV-1 disease progression. HIV-1 infection goes through three distinct stages; acute infection, chronic infection and the AIDS phase.

1.2.2.7 Elite control of HIV

A group of HIV-positive individuals, called elite controllers, are able to spontaneously control their HIV infection by suppressing the virus to undetectable levels (< 50 copies/mL by standard methods) without the requirement for ART [49]. This group, which represents less than 1% of all HIV positive subjects, is highly interesting to HIV researchers because when the mechanisms behind their control are uncovered, it will likely boost the vaccine and cure development. A genome-wide association study identified HLA class I as a major genetic determinant of elite control [50] and the HLA-B*57 allele is strongly associated with elite control [51-53]. Additionally, elite controllers display an increased $CD8^+$ T cell polyfunctionality and perforin production [54, 55]. However, elite controllers are a very heterogeneous group and the exact mechanisms behind this superior control remain unknown as do the reasons to why some elite controllers eventually lose the control of the virus.

1.2.2.8 HIV treatment

Ever since the beginning of the HIV/AIDS epidemic, research has been focused on finding a treatment or even better, a cure or a vaccine to stop the epidemic. To date, the cure and vaccine strategies have been unsuccessful. The first antiretroviral treatments against HIV were approved in 1987 when nucleoside reverse transcriptase inhibitors (NRTIs) that inhibit the function of RT were developed, however drug resistance was quickly developed. In 1995 the protease inhibitors (PI), which block the function of the viral protease and thus the maturation of viral particles were introduced and in 1996 the non-nucleoside reverse transcriptase inhibitors (NNRTIs) another drug class that inhibits the function of RT were discovered. In 1996, it was found that when combining 2 NRTIs with a NNRTI or a PI in a three-drug regimen, the virus could be successfully repressed. This was the start of a new era with combination ART, enabling HIV-infected individuals to live near to full-length lives.

Since 1996 a number of new classes of drugs have been discovered; in 2003 the fusion inhibitors and entry inhibitors were developed. These drugs block the fusion of the viral particle and the host cell (blockade of gp41) or hinder the binding of HIV to receptors of host cells (e.g. CCR5 inhibitors). In 2007, integrase inhibitors that hinder the integration of viral DNA into host DNA were introduced. Today the "standard of care" treatment is two nucleoside/nucleotide inhibitors together with a third drug, either a PI, NNRTI, integrase inhibitor or a fusion/entry inhibitor.

However, in resource-limited countries, not all HIV-infected individuals have access to ART and the treatment strategies may not always be optimal. Another concern is the increasing prevalence of acquired and transmitted drug-resistance, which may halt the progress made during the last decade [56]. Furthermore, in resource-limited settings, there is a problem with monitoring the patients, as the equipment required to measure viral loads, CD4 counts and other clinical parameters are not always available. Additionally, in many parts of the world HIV is still considered a stigma resulting in that many people do not know their HIV status. Therefore, in 2014 the UNAIDS launched a new strategy, named 90/90/90, meaning that by 2020, 90% of all HIV-infected people will know their status, 90% of the diagnosed individuals will be on ART and 90% of the treated individuals will successfully repress the virus to undetectable levels. This was recently updated to 95/95/95 to be reached in 2030. In some countries 90/90/90 is already a reality and as treatment leads to an increased life expectancy, more and more people infected with HIV live near full-length lives. However, in many parts of the world there is still progress to be made.

As the HIV-positive population is aging, physicians are faced with a new problem, which is treatment of the co-morbidities associated with long-term chronic HIV-infection. Today, the main object for the physicians is not to keep the patient alive, but to increase their quality of life [57, 58]. Even during successful treatment, HIV-positive individuals suffer from a number of co-morbidities such as cardiovascular disease and frailty syndrome which have been linked to the increased immune activation in HIV-infection [57]. Therefore, in order to stop the epidemic and limit the vast costs of HIV-treatment, a vaccine and/or a cure for HIV is still highly desirable.

1.2.2.9 Immune activation during HIV infection

During HIV infection the immune system becomes highly activated, a process that begins already during the acute phase of infection. Although a controlled immune activation is initially beneficial, a prolonged high-level immune activation is linked to disease progression [59]. The gastrointestinal tract is a major site for HIV replication and massive $CD4^+$ T cell depletion during the early phase of infection [60-63]. This damages the gut mucosa, causing leakage of microbial products from the gut into the circulation, a process known as microbial translocation [64]. Translocation of microbial products causes activation of both adaptive and innate immune cells and is highly correlated to the levels of the activation markers CD38 and HLA-DR [59] on $CD8^+$ T cells and to $CD4^+$ T cell reconstitution following initiation of ART [64]. This is further supported by studies on African green monkeys that are a natural host for

SIV, as they display little immune activation and lack of microbial translocation. However they develop immune activation when their gut is damaged by experimental methods, supporting the role of microbial translocation in immune activation [65].

Additionally, a process called pyroptosis was recently shown to be responsible for the death of abortively HIV-infected quiescent $CD4^+$ T cells in the lymphoid tissues [66]. In contrast to apoptosis, it causes the dying cells to release their cytoplasmic contents and proinflammatory cytokines, thus recruiting new cells to the site [66]. These processes together cause T cell activation, which is initially beneficial, but becomes pathogenic during the chronic phase of the disease.

1.2.2.10 Immune exhaustion during HIV infection

The process of immune exhaustion was originally described in murine models of chronic viral infection [67] and later $CD8^+$ T cell exhaustion was also described for HIV [68-71]. Exhausted $CD8^+$ T cells upregulate inhibitory receptors such as PD-1, CD160, 2B4 [68-73] and TIGIT ([73] & **Paper III**) on their surface, a process linked to decreased functionality, proliferative potential and a transcriptional profile of T-$bet^{dim}Eomes^{hi}$ ([68-71, 74] & **Paper II**) (Fig. 6). The functional properties are lost in a predetermined order. First, production of IL-2 is gone, followed by proliferative capacity and cytotoxicity. This is followed by loss of TNF production and in the later stages IFN-γ production. The very last stage of T cell exhaustion involves elimination of the exhausted cells [14] (Fig. 6). Although the functional capacity of HIV-specific $CD8^+$ T cells is suboptimal, the exhausted cells have some residual functions as HIV escapes from $CD8^+$ T cell immune pressure during chronic HIV infection [75, 76] and cells expressing PD-1 retain some functional capacity [77]. However there might be levels of exhaustion as high expression of PD-1 on $CD8^+$ T cells is associated with a more exhausted phenotype than cells expressing low levels of PD-1 [78] and accumulation of several inhibitory receptors is linked to a more dysfunctional phenotype of virus-specific $CD8^+$ T cells [71].

The process of T cell exhaustion is likely regulated at the transcriptional level, where the T-box transcription factors T-bet and Eomes have been shown to play an important role. During a chronic viral infection T-bet expression decreases whereas Eomes expression increases resulting in that the majority of the cells are $Eomes^{hi}$ [19]. Furthermore, increased expression of T-bet has been linked to a better cytotoxic potential in elite controller subjects [79].

The upregulation of inhibitory receptors likely represents a way for the immune system to limit the immunopathology caused by prolonged antigen exposure (reviewed in [1, 15], however this comes at a cost as it leads to a suboptimal control of the virus due to the exhausted state of the virus-specific $CD8^+$ T cells.

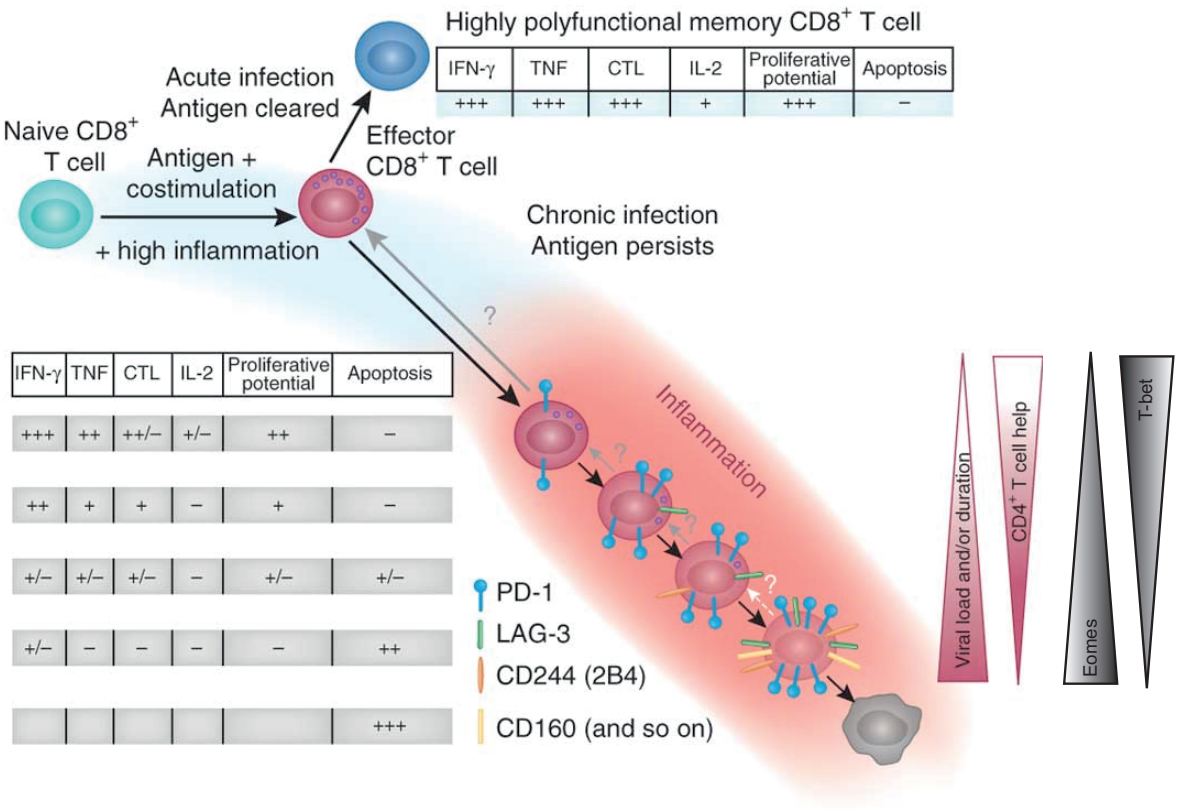

Figure 6. Immune exhaustion. A cleared viral infection results in a population of highly functional memory $CD8^+$ T cells ready to combat re-infection. However, HIV causes a chronic infection during which virus persists, causing a massive inflammation and exhaustion of the $CD8^+$ T cells. Exhausted $CD8^+$ T cells lose their functional capacity, upregulate inhibitory receptors and gain a specific transcriptional profile. Adapted from [14]. Reprinted with permission.

1.2.2.11 HIV latency

One of the largest obstacles in obtaining a cure for HIV is the occurrence of latent infection in resting $CD4^+$ T cells, resulting in a viral reservoir [80, 81]. Experiments in rhesus macaques have shown that seeding of the viral reservoir happens as early as three days after initial infection [82]. Latency is established when HIV infects a $CD4^+$ T cell that is transitioning from an active effector state into a resting memory state, which is a part of the normal physiology of T cells (reviewed in [83, 84]) but HIV has also been shown to directly infect resting $CD4^+$ T cells *in vitro* [85]. The latently infected cells do not produce any virus and will therefore not be recognized by the immune system. Furthermore, ART targets replicating virus, making it impossible to kill virus hidden in latently infected cells. Many of latently infected cells are present in secondary lymphoid tissues such as lymph nodes and GALT [80, 81] and Tfh cells have been identified as one of the major cellular compartments of latent HIV-infection [8, 86], further complicating access to the latently infected sites. Lately studies have been focusing on reactivating these resting cells in order to make the virus visible to the immune cells, a strategy named “shock-and-kill” [87] that will be discussed in section 1.3.3.

1.3 HARNESSING THE POWER OF T CELLS TO CURE DISEASE

The importance of T cells in combating disease was discussed above. In this section I will discuss the use of T cells in immunotherapy after HSCT and in HIV vaccine and cure strategies.

1.3.1. T cell immunotherapies after hematopoietic stem cell transplantation

HSCT is associated with a high risk of relapse and complications caused by opportunistic viral infections. Furthermore, the recovery of T cell immunity takes up to two years after the HSCT, during which the patients are at an increased risk of obtaining opportunistic viral infections [88]. Therefore, donor lymphocyte infusions (DLI) are used to improve the restoration of T cell immunity after HSCT. DLI involves transferring T cells from the patient or from a donor into the patient after the HSCT in order to attack pathogens and/or malignant cells. The cells may also be altered before infusion and several strategies have been developed, for example genetically modified T cells that express chimeric antigen receptors (CARs) [89], pre-activation of the cells using CD3/CD28 beads [90], expansion of T cells against a specific virus or malignancy [91, 92] and stimulation of naïve T cells with viral or tumor antigens [93]. In order to make these approaches more effective and to increase the number of cells generated by these approaches, interleukins are commonly used in T cell expansions. IL-2 has been used in vitro and in clinical studies and increases the number of T cells [94, 95], however, IL-2 also expands Tregs [96], which dampen the desired immune responses. In order to avoid this IL-7 has been used to expand the cells without expanding Tregs, resulting in promising results in pre-clinical and clinical studies [97-99].

It is of importance that the expanded cells that are transferred into the patient are of good quality and retain a functional capacity. Furthermore, the memory status of the cells is of importance, as the cells need to have effector functions as well as a capacity to form memory cells to combat re-emerging viral or tumor antigen. However, the exact fate of the injected cells in the human body is not known and therefore the capacity of T cells to form contacts with target cells and to eliminate the cells need to be further investigated in order to improve these treatment strategies.

1.3.2. HIV vaccine strategies

The optimal vaccine for HIV would be a prophylactic vaccine, however this has been difficult to develop due to the great genetic variability of HIV-1. The first HIV vaccine trials focused on creating neutralizing antibodies against HIV *env*. As T cells are the focus of this thesis, the B cell responses will not be discussed in detail, however successes have been made in the field of B cell responses against HIV as broadly neutralizing antibodies (bNAbs), able to neutralize a wide range of HIV-1 strains have been identified in a subset of HIV-infected subjects (reviewed in [100]). However the attempts to elicit these by vaccination have thus far not been successful (reviewed in [101, 102]). Many have instead looked into the T cell arm of the adaptive immune system, with the aim of creating T cell responses against viral proteins. The common goal for T cell based vaccines has been to find viral epitopes targeted by T cells

in conserved regions of the virus, i.e. regions in which escape mutations cannot occur without casing severe damage to the virus. In order to target a broad range of HIV-1 strains, viral peptides can be combined in to "mosaic" vaccines maximizing the coverage of T cell epitopes [103-105]. However, both the humoral and cellular arm of the adaptive immune system will be required in order to create a successful vaccine against HIV-1.

The 2007 STEP trial and the 2013 HVNT trials both using adenovirus 5 vectors (Ad5) to carry HIV DNA, were terminated due to an increased risk of HIV-infection in the vaccinated population [106, 107]. However, the first success, although moderate, came in 2009, when the RV144 trial, based on a canary pox vector vaccine showed a vaccine efficacy of 31%. The RV144 was found to generate antibody dependent cellular cytotoxicity (ADCC) [108] and vaccination generated both T and B cell responses suggesting that both are required for an efficient vaccine [109]. In 2011 a paper was published on the success of a vaccine against SIV based on a CMV-vector in rhesus macaques. This vaccine had a 50% efficacy in monkeys lasting more than one year after immunization [110]. Interestingly, this vaccine had a capacity to induce broad $CD8^+$ T cell responses restricted by both MHC class I and MHC class II alleles [111] and a later study showed that the broad $CD8^+$ T cell responses were restricted by the non-classical HLA class Ib molecule HLA-E [112], providing a novel approach to HIV vaccine research. However, future studies will determine whether this new vaccine strategy will be efficient in human trials.

Although a prophylactic vaccine has proven difficult to develop, important discoveries have been made on the way. Future vaccines against HIV will need to induce T cell responses against conserved regions of the virus and induce bNAbs or perhaps utilize a novel way of inducing immunity, such as the CMV-vector based vaccine strategy against SIV, as commonly used vaccination strategies have been proven unsuccessful.

1.3.3. HIV cure strategies

Since the start of the HIV epidemic, only one person, the "Berlin patient" has been cured from HIV infection [113, 114]. In this case, a HIV-infected man who developed leukemia was treated with a HSCT. The stem cell donor in this case had a homogenous deleterious mutation in the gene encoding CCR5, the main co-receptor for HIV entry, rendering the target cells to be protected from infection. The "Berlin patient" has remained completely free from detectable virus without ART and is considered cured from HIV. However, as HSCT is associated with a high risk for co-mortalities (section *1.2.1.2*) and as most HIV-positive individuals on ART are healthy HSCT is not considered a feasible cure for HIV.

Today, the shock-and-kill strategy [87] is one of the main approaches for a HIV cure. The "shock" part entails reactivating latently infected cells to start producing virus thereby making them visible for the immune system, whereas the "kill" part involves T cells and B cells that will clear the reactivated virus.

In order to "shock" latently infected cells into producing virus, latency reversing agents such as histone deacetylase inhibitors (HDACi) have been used to increase the production of virus

in infected cells *in vitro* and *in vivo,* however, their effects do not last long-term [115-119]. Another obstacle is that virus-production currently is measured in peripheral blood, whereas many latently infected cells are present in the lymphoid tissues such as in lymph nodes [120] and GALT [121], making it difficult to measure the size of the latently infected reservoir. The future of this approach requires better methods for defining the latent reservoir and to optimize the efficiency of HDACi possibly by combining them with other classes of drugs that can reverse HIV latency (reviewed in [83]).

The "kill" part is also complicated as the HIV-specific $CD8^+$ T cells are exhausted and are not able to eliminate reactivated cells. Furthermore, the virus in the latently infected cells may contain escape mutations, resulting in that $CD8^+$ T cells may not even recognize the re-emerging virus [122]. As exhausted $CD8^+$ T cells express inhibitory receptors, antibody blockade of these receptors has been developed to restore the function of HIV-specific cells. The first studies showed that blockade of PD-1 on HIV-specific $CD8^+$ T cells *ex vivo* partly restored their functional capacity [68-70], followed by blockade of inhibitory receptors such as CD160 [72] and Tim-3 [123] on HIV-specific $CD8^+$ T cells. Recently, co-blockade of PD-1/PD-L1 and TIGIT *ex vivo* was shown to restore $CD8^+$ T cell function better than single-blockade of either PD-1/PD-L1 or TIGIT on cells from HIV-infected subjects and cancer patients [73, 124, 125], suggesting that co-blockade of several inhibitory receptors is likely to be beneficial in order to restore the function of HIV-specific $CD8^+$ T cells. However, the TIGIT PD-1/PD-L1 co-blockade is not equally effective in all patients and in murine models, the level of PD-1 has been shown to impact the efficacy of PD-1/PD-L1 blockade as $PD\text{-}1^{dim}$ cells respond to treatment whereas $PD\text{-}1^{hi}$ cells do not [78] and for $CD8^+$ T cells, PD-L1 blockade efficiency is inversely correlated with viral loads [126]. Therefore the success of inhibitory receptor blockade may depend on the level of $CD8^+$ T cell exhaustion, however, further trials are required to investigate this.

Additionally, the transcriptional regulation of T cell exhaustion must be further investigated. In murine models, exhausted $CD8^+$ T cells express a transcriptional profile of increased Eomes expression and decreased expression of T-bet, where the persistent antigen exposure drives the cells toward an $Eomes^{hi}$ profile [19]. This led us to investigate the role of T-bet and Eomes on $CD8^+$ T cell exhaustion in HIV-infected subjects (**Paper II**). Additionally, T-bet is able to decrease PD-1 expression on $CD8^+$ T cells by hindering transcription of the PD-1 gene [20] whereas knockout of TIGIT increases T-bet expression on $CD4^+$ T cells [127], showing a close link between inhibitory receptors and transcription factors involved in T cell differentiation. The question remains whether the transcriptional profile can be reversed in order to restore $CD8^+$ T cell functionality.

2 AIMS

Paper I: To use a microwell chip based screening method to investigate the migration patterns and cell-cell interactions of individual T cells cultured in IL-2 or a combination of IL-2 and IL-7.

Paper II: To investigate the role of the T-box transcription factors T-bet and Eomes in $CD8^+$ T cell exhaustion during HIV-1 infection.

Paper III: To investigate the role of the TIGIT/CD226/PVR axis in $CD8^+$ T cell exhaustion during HIV-1 infection

3 MATERIALS AND METHODS

3.1 STUDY DESIGN AND PATIENT MATERIALS

The study material in **Paper I** consisted of blood samples from 15 anonymous healthy donors Karolinska University Hospital Huddinge.

In **Paper II** the patient material consisted of blood samples gathered at the HIV clinics at Karolinska University Hospital Huddinge and Venhälsan at Stockholm South General Hospital. Samples were collected from 52 individuals with chronic untreated HIV-infection and 12 HIV-infected individuals on long-term ART (> 10 years, undetectable VL for > 8 years). Out of the untreated individuals, 24 were followed longitudinally at 0, 2, 4, 6, 8, 12-16 weeks and 5-7 months after initiation of ART. Additionally, samples were collected from 20 healthy donors.

In **Paper III** blood samples were collected from HIV-infected individuals at the HIV clinics at Karolinska University Hospital Huddinge and Venhälsan at Stockholm South General Hospital. In total, 30 treatment naïve HIV-infected subjects (collected for the ACT4ART study) and 20 long-term treated (> 6 years ART, > 5 years with undetectable VL) and 26 healthy controls were collected. Additionally peripheral blood mononuclear cell (PBMC) samples were obtained from the OPTIONS [128] (subjects with acute HIV-infection) and SCOPE [129] (elite controller subjects) cohorts at San Francisco Positive Health Program, San Francisco General Hospital. Furthermore, lymph node samples and paired blood samples were collected from 8 HIV-infected subjects at the Centre for Infectious Diseases Research, National Institute of Respiratory Diseases, Mexico City. Lymph nodes and blood samples were collected from 8 HIV-negative subjects at the University of Pennsylvania as negative controls.

3.2 FLUORESCENCE MICROSCOPY

In this thesis, confocal fluorescence microscopy was used to image the actions of T cells in a microwell chip. In a fluorescence microscope one or several lasers illuminate the sample in which the structure of interest has been labeled with fluorescent dyes or antibodies conjugated to fluorescent dyes. This results in that the fluorophore emits light at a specific wavelength that is picked up by the detectors connected to the microscope. By overlaying images from several channels, it is possible to create multi-color images where the location of each marker in relation to each other can be determined. Compared to conventional fluorescent microscopy, confocal microscopy increases the optical resolution by use of a pinhole that prevents detection of out-of-focus light. This technique enables imaging of focal planes making it possible to create three-dimensional reconstructions of the imaged samples.

3.2.1 Imaging of T cells in a microwell-chip

Single cell techniques for investigation of T cells are many, however flow cytometry and conventional time-lapse microscopy do not enable long-term studies of T cell migration

patterns or cell-cell interactions. With imaging flow cytometry, combining conventional flow cytometry and fluorescent microscopy, cell-cell interactions can be imaged, however, this only provides a snapshot of the events. Furthermore, conventional time-lapse microscopy does not allow for containment of the cells within a specific area, therefore cells migrate in and out of the imaged area, resulting in that their behavior cannot be followed for extended periods of time. In order to fully understand the heterogeneity of T cells and the cellular events taking place, a microwell-chip based imaging platform, enabling containment of cells in silicon microwells has been developed in our group to allow for analysis of the actions of NK cells at the single cell level for up to 48 hours [130-132].

3.2.2 Microwell-chip

The microwell-chip used in this thesis is a 2×2 cm silicon microchip mounted on a glass slide. The chip contains 81 wells, each with a size of 450×450 µm and a well depth of 300 µm (Fig 7). This enables us to contain the cells within the wells in order to follow their migration pattern. Before use, the chip was sterilized in ethanol, after which the wells were coated with fibronectin to provide the cells with a surface suitable for cell migration. After this the chip was assembled in its holder, where it was held in place by magnets and a polydimethylsiloxane (PDMS) gasket. Finally the coating fluid was replaced with cell culture medium (Fig. 7).

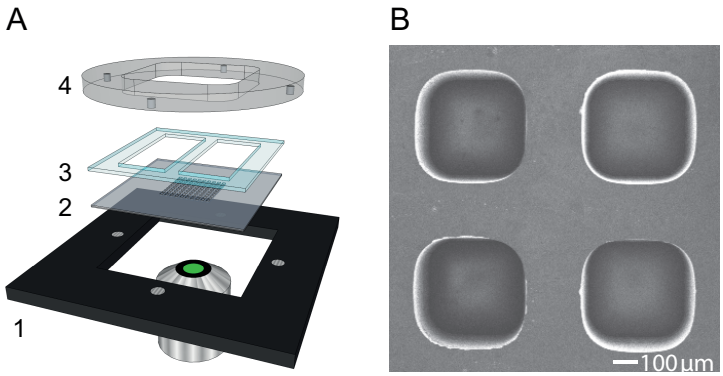

Figure 7. The microwell-chip. (A) Schematic picture of the microwell-chip setup, consisting of ; (1) custom-made metal holder, (2) microwell-chip, (3) PDMS gasket and (4) lid. (B) Scanning electron microscopy image of a subsection of the microwell-chip.

3.2.2.1 Cell culture

PBMCs were separated from blood samples using density gradient centrifugation (Lymphoprep). T cells were subsequently isolated using anti-CD3/anti-CD28 beads, after which the cells were expanded for 7 days with the beads and addition of IL-2 or IL-2 + IL-7.

3.2.2.2 Staining of cells for flow cytometry analysis

After 7 days in culture, extracellular staining of the cells was performed to measure occurrence of Tregs (CD4, CD25, CD39), γδ-T cells (TCR-γδ), memory phenotype (CD45RO, CCR7) and cell death (7-AAD).

3.2.2.3 Staining of cells for microwell-chip analysis

After 7 days in culture the cells were stained with fluorescent dyes in order to distinguish the two cell populations (effectors and targets). Calcein Green (AM) was used for target cells and Calcein Red-Orange for T cells. The target cells were seeded into the chip and when the wells contained ≈ 60 cells/well, cells that had not fallen into the wells were removed by changing the culture medium above the microwells and the remaining cells were allowed to adhere to the surface for 1 hour. Subsequently, the effector cells were seeded into the chip until ≈ 50 cells/well were obtained after which abundant cells were removed by changing the culture medium above the microwell-chip.

3.2.2.4 Time-lapse microscopy

The microwell-chip was imaged in an inverted confocal fluorescence microscope with an environmental chamber kept at 37°C, 5% CO_2 throughout the experiment. The microscope had a motorized stage allowing for automatic collection of images from selected wells at multiple positions. The pinhole was kept open to maximize the amount of light detected from the cells. For each experiment, four wells were imaged every 2 minutes for 7 hours with a 20x objective.

3.2.2.5 Image analysis

Images were analyzed with ImageJ software (US National Institutes of Health, Bethesda, MD, USA). Each cell was tracked manually and interactions with target cells were scored. Each contact lasting ≥ 2 time points was counted as a true interaction. Cell death was defined by a loss of staining and/or blebbing or swelling of the cell membrane. Cells that could not be followed for at least 60 minutes were excluded from the analysis.

3.3 FLOW CYTOMETRY

Flow cytometry is a widely used tool to rapidly investigate multiple parameters of millions of heterogeneous immune cells. In a flow cytometer, fluorescently labeled cells are transported in a sheath fluid through a laser beam one by one. When hit by the laser the fluorochromes emit light at different wavelengths that travels through a set of filters and photomultiplier tubes to the detectors (Fig. 8). The method has developed quickly in the last decade, as in the beginning stages 3-4 fluorochromes could be used simultaneously, whereas today it is theoretically possible to use up to 18 colors in the more advanced flow cytometers, even though most panels are restricted to 15-16 colors due to overlaps in the emission spectra between fluorochromes. Mass cytometry, using antibodies labeled with isotopes instead of fluorescent dyes allows for detection of up to 40 markers simultaneously (reviewed in [133]).

Here, the mass of the isotopes is measured, resulting in narrower peaks, allowing for multiple markers to be analyzed simultaneously without the risk of overlap. In this thesis, multi-color (up to 16 colors) flow cytometry has been used to measure functional and phenotypic markers of human T cells.

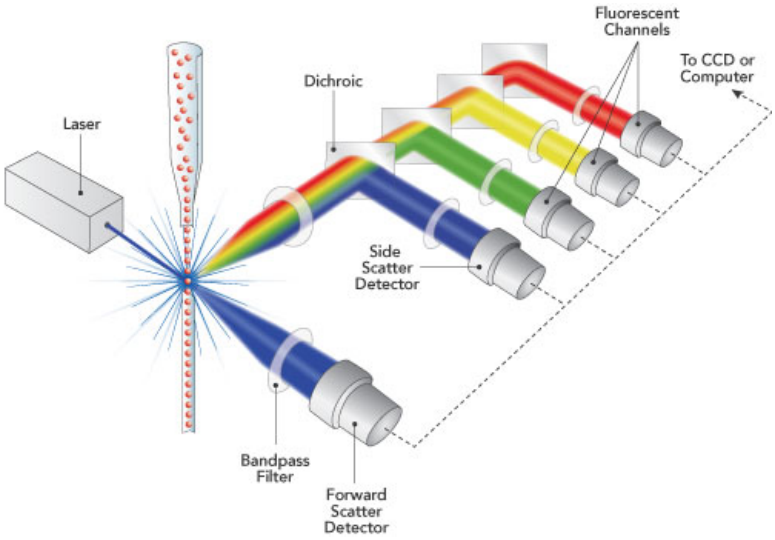

Figure 8. Image of the basic components of a flow cytometer. Fluorescently labeled cells pass through a laser beam, sending out light that passes through a set of mirrors and filters on its way to the detectors that converts the light into an electronic signal that is sent to a computer.

3.3.1 Peptide stimulations and intracellular staining of cells

In **Paper II** and **Paper III**, PBMCs (1-2x10^6/well) were stimulated with overlapping HIV Gag-p55 and HCMV pp65 peptides (15-mers overlapping by 11 amino acids at a concentration of 1 µg/mL) to detect HIV and CMV specific responses or in medium alone for negative controls in the presence of anti-CD107a antibody (detection of degranulation) and the protein transport inhibitors monensin and Brefeldin A. The cells were incubated for 10 hours at 37°C 5% CO_2 after which they were stained with extracellular antibodies and LIVE/DEAD Aqua amine dye solution. When performing extracellular staining only, cells were subsequently washed and fixated in a PBS solution containing 1% paraformaldehyde. In order to stain for cytoplasmic proteins and transcription factors, the cells were fixated and permeabilized using a FoxP3 transcription factor buffer kit, followed by staining with intracellular markers after which the cells were washed and resuspended in PBS + 1% paraformaldehyde.

3.3.2 Analysis of flow cytometry data

PBMCs and lymph node mononuclear cells (LNMCs) were analyzed on a modified 4-laser LSR Fortessa (BD Biosciences). Single-stain compensation controls were performed with antibody capture beads (BD Bioscience). Flow cytometry data was analyzed with FlowJo 8.8.7 (Treestar). Fluorescence minus one (FMO) gating strategies were use to set the manual gates when applicable. A virus-specific response was considered positive if ≥ 0.05% of the

cells produced IFN-γ after a background reduction and if the virus-specific response was larger than twice the negative background signal.

3.4 BIOINFORMATICS

In order to interpret and visualize the complex data acquired from flow cytometry analysis, bioinformatics is useful. In this thesis we have used three bioinformatics methods; SPICE (**Paper II** & **III**), principal component analysis (**Paper II**) and viSNE (**Paper III**) to analyze the data (Fig. 9).

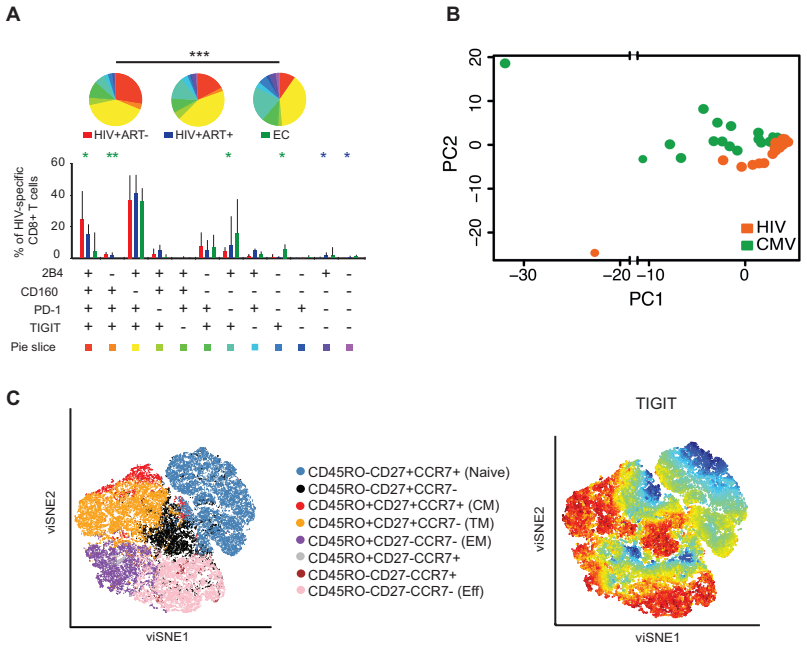

Figure 9. Bioinformatics. (A) SPICE plot depicting combinations of 4 inhibitory receptors (2B4, CD160, PD-1 & TIGIT) on HIV-specific $CD8^+$ T cells in three groups of patients (HIV+ART-, HIV+ART+ & EC). (B) PCA plot depicting HIV-specific (orange) and CMV-specific (green) $CD8^+$ T cell populations combining functional and inhibitory markers (n=256). (C) viSNE analysis of $CD8^+$ T cell maturation phenotype (left plot) and intensity of TIGIT expression on $CD8^+$ T cell maturation phenotypes (right plot).

3.4.1 SPICE

SPICE (Simplified Presentation of Incredibly Complex Evaluations) is a freely available software [134] used to present complex multi-parameter flow cytometry data in a more comprehensible manner. In **Paper II** and **Paper III,** SPICE was used to create graphs of pre-gated (Boolean gating) combinations of T cell functions and of inhibitory receptor expression and to compare these between groups.

3.4.2 Principal component analysis (PCA)

When acquiring large amounts of multivariate data, statistical methods able to reduce the dimensionality of the data are required. Principal component analysis (PCA) is an unsupervised statistical method that reduces the dimensionality of the data by projecting complex data into two dimensions while maintaining single-cell resolution. In **Paper II**, PCA was used to compare HIV- and CMV-specific $CD8^+$ T cells pre-gated (Boolean gating) for

functional and inhibitory receptor characteristics in order to see how the groups were distributed in relation to each other.

3.4.3 viSNE

viSNE is another tool for visualization of multivariate single-cell data, in this case based on the t-Distributed Stochastic Neighbor Embedding (t-SNE) algorithm. viSNE interprets complex single-cell data in order to find the best two-dimensional representation while preserving the local and global geometry of the data [135]. In **Paper III**, viSNE was used for plotting the expression pattern and intensity of inhibitory/stimulatory receptors on naïve and memory subsets of $CD8^+$ T cells.

3.5 STATISTICS

In all papers, statistical analysis was performed using either GraphPad Prism, Matlab, R environment or SPICE software. Mann-Whitney U test was used to compare two unmatched groups of samples and Wilcoxon-matched pairs signed rank test was used to compare two matched groups of samples.

Additionally, in **Paper I**, Chi-square test was used to evaluate cell-cell contacts, cell division and cell death. In **Paper II** and **Paper III**, correlation analysis was done using Spearman rank tests. One-way ANOVA, followed by Kruskall-Wallis non parametric Dunn's multiple comparison tests were performed to compare 3 or more groups and permutations tests were performed on data from SPICE. In **Paper II**, data from Boolean combinations were analyzed by PCA. The Kolmogorov-Smirnov test was used to test the null hypothesis that the groups from PCA were drawn from the same distribution. In **Paper III** viSNE analysis was performed in order to visualize complex expression patterns of cell surface markers on $CD8^+$ T cell subsets.

4 RESULTS AND DISCUSSION

The main aim of this work was to gain a deeper knowledge of human T cell functions, such as migration, cell-cell interactions, memory differentiation and cytotoxic capacity and of the mechanisms that impair these properties. In this thesis two single-cell analysis methods have been used to investigate the functional properties of human T cells; imaging in a microwell-chip and multi-color flow cytometry. This section will present and discuss the results from these studies.

4.1 MICROWELL CHIP BASED IMAGING OF T CELLS CULTURED WITH IL-2 OR IL-2+IL-7

After a HSCT patients are at risk for relapse and acquiring opportunistic viral infections. DLI of *ex vivo* or modified T cells can be used to aid in the recovery of T cell immunity in order to decrease the risk of complications. However, there is a need to speed up the expansion process and to improve the safety of the protocols. In **Paper I,** a novel microchip based screening method was used to monitor the migration and cell-cell interaction patterns of T cells expanded in two different conditions; IL-2 alone or a combination of IL-2 and IL-7.

Cells from healthy blood donors were expanded for 7 days together with anti-CD3 and anti-CD28 beads in order to induce cell expansion. In addition, IL-2 alone or in combination with IL-7 was added to the cells. After 7 days the cells were stained for flow cytometry analysis or microwell-chip based imaging.

In 7 out of 12 subjects addition of IL-7 gave a higher fold expansion compared to IL-2 alone however, this was not statistically significant. These data were not in line with previous data based on cord-blood derived cells, where addition of IL-7 resulted in a significant increase in expansion compared to IL-2 alone [136]. The difference might be due to different glycosylation patterns of IL-7 as we used CYT107a produced in the CHO cell line whereas Berglund et al. [136] used IL-7 produced in *E. coli*. Furthermore, cord-blood derived T cells have a more naïve phenotype than PBMCs from adult subjects, which may alter the response to cytokine exposure. The flow cytometry data did not show any differences between the groups in terms of T cell maturation phenotypes. From the microwell-chip data we found that neither the migration speed nor the number or the duration of cell-cell interactions differed between cells cultured in IL-2 + IL-7 compared to cells treated with IL-2 only. Interestingly, addition of IL-7 significantly reduced cell death in the microwell-chip, although it had no effect on mitosis, suggesting that IL-7 renders the cells more resistant to cell death. However, this could not be corroborated by flow cytometry, where few dead cells were found. The differences might be a result of the definition of cell death as in the microscope, cell death was defined as a blebbing or swelling of the cell membrane, whereas in the flow cytometry analysis cell death was measured by 7-AAD and Annexin V. Additionally, in the microwells dying cells are imaged continuously whereas dying cells in flow cytometry analysis may be disintegrated resulting in that they are lost during wash steps or that they due to their small size are discarded as cellular debris.

In summary these data show that IL-7 in combination of IL-2 is beneficial when preparing T cells for immunotherapy and that the microwell-chip based method is suitable for monitoring of T cells.

4.2 FLOW CYTOMETRY ANALYSIS OF HIV-SPECIFIC CD8$^+$ T CELLS

During the course of HIV-1 infection CD8$^+$ T cells become exhausted due to the constant antigen burden, resulting in a loss of functional capacity and an upregulation of inhibitory receptors. This process that has been investigated in **Paper II** and **Paper III**.

In murine models the transcription factors T-bet and Eomes are differentially expressed in exhausted and functional CD8$^+$ T cells [19], however their role in exhaustion of human HIV-specific cells is not known. Therefore, in **Paper II**, we investigated whether T-bet and Eomes are transcriptionally linked to CD8$^+$ T cell exhaustion in HIV-1 infection.

Samples from treatment-naïve HIV-infected subjects and long-term treated HIV-infected subjects were analyzed with flow cytometry. We found that T-bet and Eomes were inversely expressed on CD8$^+$ T cells in HIV-infected subjects, resulting in a T-bet^{dim}Eomes^{hi} profile that was linked to single- and triple expression of the inhibitory markers PD-1, CD160 and 2B4 on bulk and HIV-specific CD8$^+$ T cells. The vast majority of HIV-specific CD8$^+$ T cells were T-bet^{dim}Eomes^{hi} whereas CMV-specific CD8$^+$ T cells in the same subjects had a more balanced expression of T-bet and Eomes. Furthermore, the frequency of T-bet^{dim}Eomes^{hi} cells was negatively correlated with the polyfunctional capacity (IFN-γ, TNF, CD107a, Granzyme B & IL-2) of the cells. Subjects initiating ART were followed longitudinally for 6 months, however the expression of inhibitory receptors and the transcriptional profile of T-bet^{dim}Eomes^{hi} remained elevated even after successful ART. In patients that had been on ART for more than 10 years, the expression of T-bet^{dim}Eomes^{hi} cells was significantly decreased compared to short-term treated subjects, however not to the levels seen for CMV-specific cells in the same subjects.

The T-bet^{dim}Eomes^{hi} profile remained even after long-term ART, suggesting that this exhausted transcriptional profile is imprinted on the HIV-specific cells and is not reverted by removing the antigen. Approaches to restore functionality to HIV-specific CD8$^+$ T cells will likely require modulation of T-bet and/or Eomes expression, as T-bet^{hi} cells are associated with a better cytotoxic capacity [79] and T-bet promotes transcription of Granzyme B and perforin in T cells [137], whereas Eomes is highly expressed in exhausted and more early differentiated T cells **(Paper II)**. Recently, in a murine model of cancer, inhibitory receptor co-blockade induced T-bet expression on tumor-infiltrating CD8$^+$ T cells [138], showing that the transcriptional profile can be reversed. Furthermore, this T-bet upregulation was associated with differentiation to an effector phenotype and IFN-γ production by tumor-specific CD8$^+$ T cells [138], however, additional studies are required to confirm this effect on human CD8$^+$ T cells. Additionally, T-bet has been shown to bind the PD-1 promoter region and is able to repress PD-1 expression in mice [20], and T-bet expression was induced by blockade of TIGIT on human CD4$^+$ T cells, suggesting that these processes can be altered by

treatment. In summary, **Paper II** demonstrates that $CD8^+$ T cell exhaustion is driven at the transcriptional level and partly explains why HIV-specific $CD8^+$ T cells cannot fully control viral replication without the presence of ART, suggesting that modulation of T-bet and/or Eomes expression is likely required to reinvigorate HIV-specific $CD8^+$ T cell responses.

The upregulation of inhibitory receptors on $CD8^+$ T cells is linked to a loss of cytokine production, cytotoxicity and proliferative capacity. Therefore, in **Paper III,** we continued the investigation of the effects of inhibitory receptor expression on HIV-specific $CD8^+$ T cell responses by investigating the role of the inhibitory receptor TIGIT in HIV-infected subjects.

Samples from treatment-naïve HIV-positive subjects, HIV-positive subjects on long-term ART, acutely infected subjects and elite controllers were analyzed with flow cytometry. We found an increased expression of TIGIT on bulk and HIV-specific $CD8^+$ T cells that was linked to expression of PD-1, CD160 and 2B4. On HIV-specific cells the increase in TIGIT expression was coupled to a decreased expression of the co-stimulatory receptor CD226. Furthermore, a high level of TIGIT expression ($TIGIT^{hi}$) was less common in elite controller subjects, compared to treatment naïve and long-term treated subjects and the $TIGIT^{hi}$ cells were linked to a decreased functional capacity (IFN-γ, TNF, granzyme B & CD107a). Additionally, $TIGIT^{hi}$ cells had a transcriptional profile of $T\text{-}bet^{dim}Eomes^{hi}$, which in **Paper II** was linked to a highly exhausted phenotype. As TIGIT needs to bind its ligand, PVR, in order to inhibit T cell function, we next measured PVR expression on $CD4^+$ T cells in blood and lymph node. PVR expression was elevated on $CD4^+$ T cells, especially Tfh cells, in HIV-infected subjects compared to healthy controls both in lymph node and blood.

The TIGIT/CD226 receptor pair shares many qualities with the CTLA-4/CD28 inhibitory/stimulatory receptor pair. Both CTLA-4 and TIGIT have a higher affinity for binding the ligands CD80/CD86 and PVR, than their respective complementary co-stimulatory receptors CD28 and CD226 and thus outcompetes them in binding capacity [16, 139]. In addition, both CTLA-4 and TIGIT can downregulate the expression of their respective co-stimulatory receptors, dampening the activating signals [125, 140], confirming the importance of the TIGIT/CD226 axis on $CD8^+$ T cell function. Blockade of inhibitory receptors such as PD-1, CD160 and TIGIT using antibodies has been shown to increase the functional capacity of HIV-specific cells [68-70, 72, 73], suggesting a potential mechanism to restore the function of exhausted $CD8^+$ T cells. However, the increased frequency of $TIGIT^{hi}$ cells among the HIV-specific cells suggests that the increased expression of TIGIT might make restoration of their functional capacity problematic. In murine models, $PD\text{-}1^{hi}$ cells do not respond to PD-1/PD-L1 blockade, whereas $PD\text{-}1^{dim}$ cells do [78], suggesting that an increased expression of inhibitory receptors, likely due to prolonged antigen-exposure drives the cells towards a highly exhausted phenotype. Interestingly, expression of the TIGIT/CD226 ligand, PVR, was increased on Tfh cells, which are one of the major sources of productive and latent HIV-1 infection [8], suggesting that PVR is upregulated on HIV-infected $CD4^+$ T cells. As $CD8^+$ T cells have a very restricted access to the B cell follicles where Tfh cells reside [141], it is likely they cannot reach the infected cells. In a recent study

in elite controller rhesus macaques the B cell follicles were the only sites in the lymph node with active HIV replication, likely due to that SIV-specific $CD8^+$ T cells could not reach these areas [142]. Additionally, our data suggests that even if $CD8^+$ T cells are able to enter the B cell follicles, they might not be able to eliminate infected cells due to the upregulation of TIGIT and PVR during HIV infection, as studies of NK cells have shown that TIGIT/PVR binding likely hinders their cytotoxic capacity [143]. In summary, our data provides evidence that the altered TIGIT/CD226/PVR axis needs to be considered for future approaches aimed at restoring the function of HIV-specific $CD8^+$ T cells.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis, single cell methods have been used to monitor the actions of human T cells with the aim to understand the mechanisms of T cell function/dysfunction.

Because of their heterogeneous nature, single cell studies of T cells are of importance to understand their functions. In **Paper I**, a microwell chip based method enabling single cell tracking of T cell migration was used. In **Paper II and III**, multi-color flow cytometry was used to measure function, phenotype, level of immune activation/exhaustion, and transcriptional activity on individual $CD8^+$ T cells.

In **paper I**, we found a decrease in cell death of T cells that were expanded with a combination of IL-2 and IL-7 when cultured in a microwell chip. These findings suggest that addition of IL-7 to the current T cell expansion protocol would make the cells more resistant to cell death. Furthermore, this study shows that the microwell chip is well suited for analysis of human T cells. As methods for imaging the behavior of human cells *in vivo* are not available, there is a need for *in vitro* models in which the behavior of human T cells can be tracked. T cells have been imaged *in vivo* in murine models (reviewed in [144]), providing important information on the behavior of T cells, however mice are not men, and although many similarities exist, we cannot be certain that what is seen in mice is true for humans. Therefore, filling the microwell-chip with a gel mimicking human tissue would allow for imaging of cell migration in vitro within an environment that mimics conditions found in the human body. Although this would also be a model, it might provide new insights into human T cell behavior and allow us to use a more controlled platform, which is easily altered, allowing for comparison of several conditions simultaneously.

In **Paper II** and **Paper III**, the exhausted phenotype of $CD8^+$ HIV-specific cells was associated with a decreased functional capacity of the cells and a transcriptional phenotype of T-$bet^{dim}$$Eomes^{hi}$. The results from **Paper II** show that even in HIV-infected subjects on long-time ART, the inverse transcriptional profile of T-bet and Eomes does not return to levels seen in CMV-specific cells. Reversal of the T-$bet^{dim}$$Eomes^{hi}$ transcriptional profile of exhausted $CD8^+$ T cells would require induction of T-bet expression, while decreasing expression of Eomes. Future studies will need to further investigate the synergistic effects of co-blockade of inhibitory receptors such as TIGIT, PD-1, CD160 and 2B4 on $CD8^+$ T cell function and phenotype. Furthermore, the effects of inhibitory receptor co-blockade on the transcriptional profile need to be assessed. Additionally, the phenotype and functional characteristics of $CD8^+$ Tcells that are restored by such treatment need to be investigated, as highly exhausted HIV-specific cells expressing high levels of inhibitory receptors ([70] & **Paper II & Paper III)** may not respond to treatment as seen for murine PD-1^{hi} cells [78].

In **Paper III**, the increased expression of TIGIT on HIV-specific cells was linked to a downregulation of CD226 on HIV-specific $CD8^+$ T cells and an upregulation of PVR on Tfh cells. Additional studies will need to investigate whether $TIGIT^+$ HIV-specific $CD8^+$ T cells

are capable of eliminating HIV-infected cells with an increased expression of PVR, as their interaction may hinder $CD8^+$ T cell cytotoxicity. The cytotoxic capacity of $TIGIT^+$ cells may be restored by antibody-blockade of TIGIT in combination with other inhibitory receptors (as discussed in the results and discussion section). Furthermore, as the B cell follicles are a major reservoir for latent and productive HIV infection, future studies will need to further investigate the presence and function of the few $CXCR5^+$ $CD8^+$ T cells in the B cell follicles and to find a way for additional HIV-specific $CD8^+$ T cells to gain access to the B cell follicles. Furthermore, the safety of such a procedure needs to be assessed in order to avoid cytotoxicity against non-HIV-infected cells.

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